J. Vet. Malaysia (2014) 26 (1):1-7

Journal Articles

MOLECULAR DETECTION OF *MYCOPLASMA GALLISEPTICUM* BY REAL TIME PCR

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SUMMARY

Mycoplasma gallisepticum (MG) causes chronic respiratory disease leading to huge economic losses to the poultry industry worldwide. Early and efficient detection is therefore crucial in reducing the loss sustained by poultry farmers and poultry industry at large. Three main approaches are used for the diagnosis of MG: isolation and identification, serology and molecular detection method. Recently, real time polymerase chain reaction has been developed for the detection of infectious organisms, but so far only a limited number of diagnostic real time PCRs have been proposed for MG. This study was carried out to develop a SYBR green real time PCR assay for the detection of MG using primer set specific to the *gapA* gene. The primer set was able to amplify the expected DNA fragment of 505 bp. The assay was found to be specific and highly sensitive in detecting MG as indicated by its ability to detect between 260 ng/µl to 26 pg/µl DNA template. In conclusion, this study successfully developed a specific and sensitive real time PCR assay for the rapid detection of MG compared to conventional PCR method. Although the cost to carry out real time PCR is more expensive, it is a more specific, sensitive, and rapid method for detection of MG as compared with conventional PCR.

Key words: Mycoplasma gallisepticum, PCR, SYBR green real time PCR, gapA gene.

INTRODUCTION

Mycoplasma gallisepticum (MG), which has a worldwide distribution, is the most economically important pathogenic avian Mycoplasma. This is a primary pathogen which can cause acute and chronic diseases leading to wide-ranging complications (Levisohn and Kleven, 2000). MG can be diagnosed using three by methods including isolation and identification of the organism, detection of specific antibodies and detection of its DNA (Bradbury, 2001). Isolation and identification is very time consuming and quite difficult due to the fastidious nature of Mycoplasma. On the other hand, serological tests were found to be non specific and unconvincing (Avakian *et al*., 1988) due to the possibility of cross reaction (Kleven *et al*., 1988).

To overcome the problem of isolation identification and serological method, molecular detection method has been developed. There are many reports regarding the detection of DNA and ribosomal RNA gene probes for MG diagnosis (Garcia *et al*., 1996), but polymerase chain reaction (PCR) based methods are more suitable due to its simplicity, rapidity, sensitivity and specificity (Harasawa *et al.,* 2004). For the concurrent detection of various organisms, multiplex PCR protocols have been defined (Mardassi *et al*., 2005). PCR based methods give positive or negative results within a day without the presence of live organism. Recently, real time PCR has been developed, which is more specific, sensitive and rapid detection method in comparison with conventional PCR although it is more expensive. Garcia *et al*. (2005) showed that, *gapA* gene is more conserved than *16S rRNA*, *mgc2* or *lp* for the detection of MG by conventional PCR, but no report was found regarding SYBR Green real time PCR using *gapA* gene for the detection of MG. Therefore, the objectives of this study were to develop a SYBR Green real time PCR assay for the detection of MG and to detect the presence of MG in commercial and village chicken flock using the developed real time PCR.

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MATERIALS AND METHODS

Sample Size

Samples were collected by using sterile cotton swabs from broilers, layers and from village chickens in the year of 2010–2012 from different states in Peninsular Malaysia. Swab samples were collected from choanal cleft and chilled on ice for 1–2 h until arrival at the laboratory. The swabs were then kept in eppendorf tubes containing 1 ml PBS at 4°C overnight and then at -20°C until DNA extraction. The sampling size, sampling location and vaccination history are shown in Table 1.

DNA Extraction

An ethanol-cleaned forcep was used to remove the cotton swab from the PBS under sterile condition. Conventional salt-based method was used to extract genomic DNA with some modifications (OIE, 2004). After extraction the DNA samples were re-suspended in 30 µl of nuclease free water, then kept at 4^0C for 30 min and finally stored at -20^0C to be later used for PCR.

Real Time Polymerase Chain Reaction (PCR) targeting the gapA gene

The SYBR Green I real time PCR amplification was carried out using the CFX96 Real Time PCR System (Bio-Rad, USA). Real time PCR was also developed for MG detection using different type of genes; *16s rRNA*, *MGA 0319*, *lp*, *mgc2* and *pvpA* gene (Callison *et al.,* 2006;

Carli and Eyigor, 2003; Mekkes and Feberwee, 2005; Raviv, Callison *et al.,* 2008; Raviv and Kleven, 2009). The primer set used in this study was targeted against the adherence protein A gene (*gapA*) of MG. Primer name, sequences, PCR product size and optimal annealing temperature are shown in Table 2. The primers were purchased from AIT biotech, Singapore. This gene was considered as one of the important conserved cytadhesion gene and there was no secondary copy of this gene. The primer used in this study was specific in the detection of MG and this gene has no size polymorphism (Tan, 2008).

Optimisation was carried out by varying PCR conditions such as DNA concentration, amplification cycle numbers and primer annealing temperature. The reaction mixture was subjected to 94^0C for 5 min as an initial denaturation, and followed by 40 cycles of denaturation at 94^0C for 20 sec, primer annealing at 60^0C for 30 sec and extension at 72^0C for 30 sec.

Table 1. Sampling size, sampling location and vaccination history of layer breeder, broiler breeder, broiler chicken and village chicken used in this study

Farm No.	Farm ID	Farming type	Vaccination	State	No. of collected samples
1	SMF1A	Layer breeder	Yes	Melaka	15
2	SMF1B	Layer breeder	Yes	Melaka	15
3	SMF5B	Layer breeder	Yes	Melaka	15
4	SMF2C	Layer breeder	Yes	Melaka	15
5	TTJF1	Broiler chicken	Yes	Melaka	15
6	TTJF2	Broiler chicken	Yes	Melaka	15
7	SM4	Broiler chicken	Yes	Melaka	15
8	SM5	Broiler chicken	Yes	Melaka	15
9	G5	Layer breeder	Yes	Melaka	20
10	SMF3	Layer breeder	Yes	Melaka	15
11	JSF1	Broiler chicken	Yes	Johor	30
12	JSF2	Broiler chicken	Yes	Johor	30
13	HL	Village chicken	No	Selangor	10
14	Tanj	Broiler chicken	Yes	Selangor	30
15	RNS	Village chicken	No	Negeri Sembilan	24
16	KS	Broiler breeder	Yes	Selangor	21
		Total			300

Table 2. The primer sequences, PCR product size and optimal annealing temperature used in this study

Primer	Gene	Sequence 5' to 3'	Location Genebank sequence	PCR product size (bp)	Optimal annealing temperature ^{0}C
gapA 5F	Adherence Protein A (*gapA*)	TCARCGTTTCTAAGATTCCTTTTG	3696 - 3719	505	60
gapA 6R		GCATCAAAACCAGTAAATTCTTGG	4177 - 4200		

Reference: García et al., 2005; Zahraa et al., 2011

Melting Curve Analysis of the Amplified PCR Products

Upon completion of the amplification, the specificity of the amplified product was confirmed by melting curve analysis whereby the reaction was incubated by raising the incubation temperature from 70^0C to 95^0C in 0.5^0C increments with a hold of 5 sec at each increment. The SYBR Green I fluorescence (F) was measured continuously during the heating period and the signal was plotted against temperature (T) to produce a melting curve for each sample. The melting peaks were then generated by plotting the negative derivative of the fluorescence over temperature versus the temperature (-dF/dT versus T) (Table 3).

Table 3. Threshold cycle (Cq) of avian Mycoplasma species which has been tested by real time PCR

Avian Mycoplasma species	Threshold cycle (Cq)	Melting temperature (Tm) ^{0}C
M. gallisepticum (MGS6)	26.05	76.5
M. inners	N/D	-
M. cloacale 383	37.22	75.5
M. meleagridis	39.18	-
WVU 1853 (*M. synoviae*)	N/D	-
M. gallinarum	36.86	75.5
M. gallinaceum	35.81	76.0
M. iowae K285 I	35.75	75.5
M. iowae K285 J	35.69	75.5
M. iowae K285 K	37.25	75.5
M. iowae K285 N	35.56	75.5
M. iowae K285 Q	36.32	75.5
M. iowae K285 R	38.37	72.5
M. immitans	36.42	75.5
M. anatis	37.01	75.5
Acholeplasmalaidlawati	N/D	-
M. columborale	35.41	75.5
M. lipofaciens	N/D	72.5
M. glycophylum	39.60	72.5
M. galopavonis	N/D	-
M. columbinasale	37.91	75.0

ND= Not Detectable

PCR Sensitivity

DNA was extracted from MG reference strain also. The extracted DNA was serially diluted in 10 fold dilutions and then amplified by the real time PCR protocol as stated above to determine the detection limit and amplification sensitivity.

PCR Specificity

Twenty avian Mycoplasma species together with reference strain, MGS6 were tested by the developed real time PCR protocol to identify PCR specificity. The Mycoplasma species other than MG used in this study were *M. inners, M. cloacale* 383, *M. meleagridis*, WVU 1853, *M. gallinarum, M. gallinaceum, M iowae* K285 I, J, K, N, Q, R, *M. immitans, M. anatis, Acholeplasmalaidlawati, M. columborale, M. lipofaciens, M. glycophylum, M. galopavonis*and *M. columbinasale.*

RESULTS

PCR Sensitivity

Three independent runs of each reaction using 10 fold serial dilutions of the reference strain, MGS6, determined the standard curve. The detection limit was 260 ng/µl to 26 pg/µl, R^2 value = 0.997 and E = 94.1%. This detection method was highly sensitive as it can detect as little as 26 pg/µl of DNA template per reaction (Figure 1).

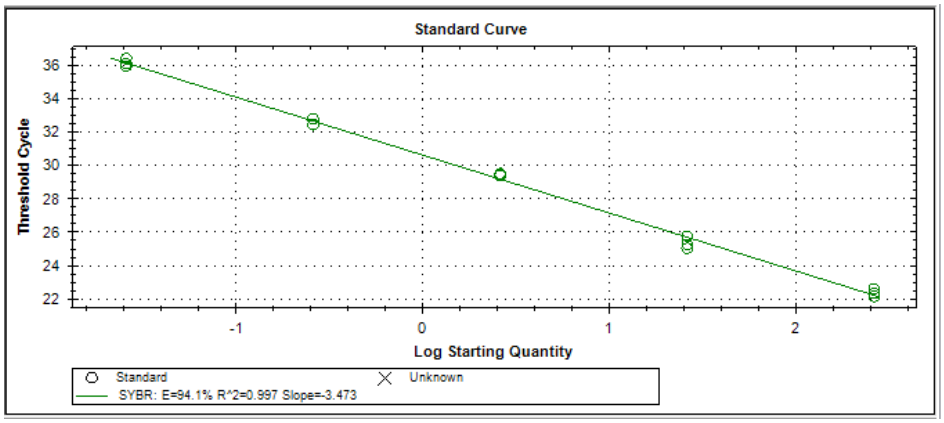

Figure 1. Amplification sensitivity and R^2 value of MG reference strain, MGS6 based on the standard curve

Table 4. List of field strains screened by real time PCR

Farm No.	Farm ID.	Farming type	Vaccination	State	No. of collected samples	No. of positive samples by real time PCR
1	SMF1A	Layer breeder	Yes	Melaka	15	14
2	SMF1B	Layer breeder	Yes	Melaka	15	15
3	SMF5B	Layer breeder	Yes	Melaka	15	8
4	SMF2C	Layer breeder	Yes	Melaka	15	1
5	TTJF1	Broiler chicken	Yes	Melaka	15	3
6	TTJF2	Broiler chicken	Yes	Melaka	15	-
7	SM4	Broiler chicken	Yes	Melaka	15	2
8	SM5	Broiler chicken	Yes	Melaka	15	1
9	G5	Layer breeder	Yes	Melaka	20	2
10	SMF3	Layer breeder	Yes	Melaka	15	3
11	JSF1	Broiler chicken	Yes	Johor	30	28
12	JSF2	Broiler chicken	Yes	Johor	30	15
13	HL	Village chicken	No	Selangor	10	1
14	Tanj	Broiler chicken	Yes	Selangor	30	1
15	RNS	Village chicken	No	Negeri Sembilan	24	-
16	KS	Broiler breeder	Yes	Selangor	21	-
		Total			300	94

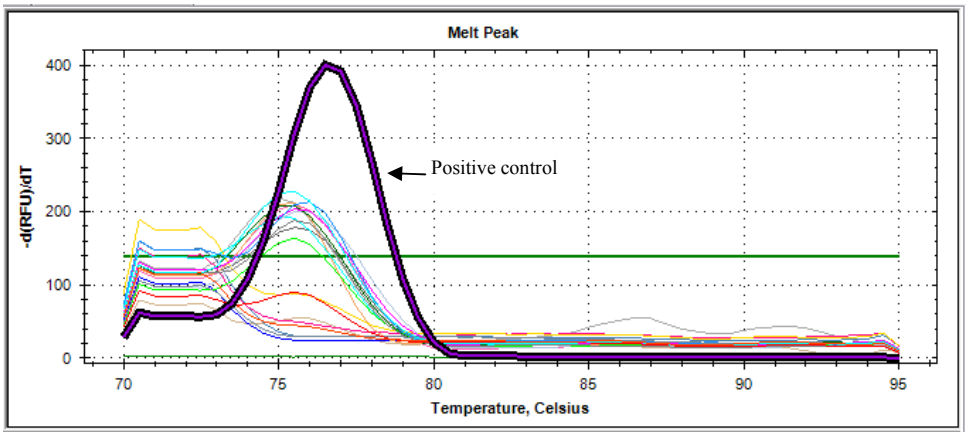

Figure 2. Melting curve of MGS6 and other avian Mycoplasma species

PCR Specificity

The specificity of this real time PCR was verified by testing with the extracted DNA of 20 other avian Mycoplasma species and also the MGS6 as reference strain. The result showed that, only MGS6 DNA was amplified at the early stage of amplification with the Cq value of 26.05. There was no amplification for *M. inners*, *WVU 1853*, *Acholeplasmalaidlawati, M. lipofaciens* and *M. galopavonis* and the amplification of other isolates were only observed after 35 cycles with Cq values ranging from 35.41 to 39.60 (Table 4). On the other hand, the melting curve analysis on MGS6 revealed a sharp and distinct peak at 76.5^0C. No melting peaks were observed for other Mycoplasma species (Figure 2). The Cq value depends on the abundance of the DNA being amplified, the more abundant the lesser the Cq value. The melting temperature is the better parameter for specificity as despite low or high DNA abundance, the melting temperature must be 76.5^0C compared to all the other isolates.

Screening of field strains

DNA of 300 field strain samples, which were collected from layer breeder, broiler chicken, broiler breeder and village chicken from different states of Malaysia, were tested by the developed real time PCR. In this study, forty-three samples were positive out of 95 samples collected from layer breeder and the prevalence rate was 45%. Fifty samples were positive out of 150 samples collected from broiler chicken and the prevalence rate was 33%. One sample was positive out of 34 samples collected from village chicken and the prevalence rate was 3%, and there was no positive sample out of 21 samples collected from broiler breeder. The overall prevalence rate was 31%, where 94 samples were positive out of 300 samples.

DISCUSSION

In this study, a real time PCR assay was developed using SYBR green dye, which was very useful for the detection and quantification of MG directly from clinical samples as it showed high sensitivity and very low detection limit. The specificity of this method was also high when tested together with 20 other avian Mycoplasma species. Following the development of this detection method, extracted DNAs of 300 samples collected from commercial broiler, layer as well as village chickens were tested for the presence of MG. Samples were collected from different states in Malaysia to fulfil the screening achievement. Results revealed a high prevalence, as most of the farms tested were positive for MG.

For the detection of MG, real time PCR method using *gapA* 5F+6R primer set of the *gapA* gene developed in this study was beneficial since it is sensitive, specific and rapid. The R^2 value was 0.997, E = 94.1% and the detection limit was 260 ng/µl to 26 pg/µl, which was a clear indication of the high sensitivity of this protocol. Moreover, a total of 20 other avian Mycoplasma species was tested by this protocol and only MGS6 (reference strain of MG) showed early amplification with 26.05 Cq value. Whereas *Mycoplasma inners*, *WVU 1853* (*Mycoplasma synoviae*), *Acholeplasmalaidlawati*, *Mycoplasma lipofaciens* and *Mycoplasma galopavonis* showed no amplification; and other avian Mycoplasma species were amplified much later at a Cq value >35, which proved that this real time PCR method was highly specific for the detection of MG. This was also a rapid detection method for MG as it gave result within an hour in comparison with the conventional PCR where other studies showed that detection of MG by conventional PCR using the gapA 5F+6R primer set of gapA gene took 3 hr and also post PCR processing was required (Zahraa *et al.*, 2011). In 2008, SYBR green real time PCR assay was also developed using *gapA* gene which could detect MG vaccine strains ts 11 and 6/85, but it could not detect pathogenic challenge strains R, R_{low} or S6 as well as field strains (Evans and Leigh, 2008). Another study showed that, a real time PCR assay using a taqman labeled probe was developed and validated targeting the *MGA 0319* gene for the detection of MG (Callison *et al.,* 2006) however, this assay is costly. Real time PCR also has been developed for MG detection using *lp* gene (Carli and Eyigor, 2003) and *mgc2, MGA 0319* and *pvpA* gene which showed high specificity and sensitivity but was limited for the known combination of strains and was not able to detect field strains (Raviv *et al*., 2008). Although the present study developed a good detection method for MG, it was not able to differentiate among MG strains.

Following the determination of sensitivity and specificity of real time PCR method developed in this study, a total of 300 local field samples was screened to determine the presence of MG. Samples were collected from layer breeders, broiler chickens, broiler breeders and village chickens from different states in Peninsular Malaysia. The results of screening by real time PCR indicated a high prevalence rate of MG in Malaysia although the farmer carried out vaccination program in commercial poultry farms. In this study, 43 samples were positive out of 95 samples collected from layer breeders, and the prevalence rate was 45%. Fifty samples were positive out of 150 samples collected from broiler chickens and the prevalence rate was 33%, 1 sample was positive out of 34 samples collected from village chickens, and the prevalence rate was 3% and there was no positive sample out of 21 samples collected from broiler breeders. The overall prevalence rate was 31%, while 94 samples were positive out of 300 samples, indicating a high prevalence rate of MG in Malaysia. This study agrees with previous studies, which indicated the high prevalence rate of MG in Malaysia (Ganapathy *et al.*, 2001; Mutalib *et al.*, 2001). In another study, in commercial chicken farms and their progeny, the total prevalence rate was 18% (Zahraa *et al*., 2011). Although these farms stated in this study had vaccination and treatment history, the high presence of MG may be due to the horizontal transmission; through infected birds, eggs, wild birds, vehicles or fomites (Jordan, 1985) or could be due to the vaccine strain also as some chickens were vaccinated by live vaccination. Other probable factors

include the stress conditions leading to reduced immunity against MG infection, poor management and cold during the rainy season. Poor ventilation, litter contamination, lack of movement restriction, are also the additional factors that contribute to MG infection (Dulali, 2003).

From this study, it can be speculated that, occurrence of MG was more in layer breeders than broiler chickens, broiler breeders and village chickens, and the occurrence of MG in broiler chickens was second highest among all. The high occurrence of MG in layer breeders might be related with bacterin used to produce vaccines, as some study showed that although bacterins contributed in the reduction of MG infection, they are not able to eliminate MG (Yagihashi *et al.*, 1992; Yoder and Hopkins, 1985), and usually have minimal value on multiple-age production sites, in case of long-term control of infection (Levisohn and Kleven, 2000). Researchers have also found that, vaccination by bacterins will not lessen the horizontal transmission of MG between layer chickens (Feberwee *et al*., 2006). Another possible reason related with high occurrence of MG is substandard hygienic conditions, poor health management and also may be lack of improved husbandry skills (Farooq *et al.*, 2002; Usman and Diarra, 2008). However, another study showed that, broiler chickens are more susceptible than village chickens and commercial layer chickens (Kartini, 2012). Although these farms had the history of fulfilment of the vaccination program, it showed similar findings with other studies. It was indicated that a small occurrence of the disease in broiler breeders can cause massive dissemination of the pathogen in commercial broiler production, and it remains in the flock constantly, as a sub-clinical form, where the bird becomes a carrier of the pathogen for the whole life (Kartini, 2012). The occurrence of MG in village chicken was very low, which may be due to the natural immunity developed in village chicken. However, there was no occurrence of MG in broiler breeders, and it may be due to the strict bio security management and vaccination program practised by the farmer.

CONCLUSION

Finally, this study indicated that real time PCR by using gapA 5F+6R primer set of *gapA* gene is highly beneficial for the detection of MG on the aspect of rapidity, sensitivity and specificity and this study also showed that MG still persists in commercial poultry. So, it is highly recommended that, the outbreak of mycoplasmosis in Malaysia needs to be controlled by the new prevention methods based on medications and vaccinations, and also probably using new vaccines based on characteristics of local MG strains.

ACKNOWLEDGEMENTS

This research was conducted under the financial support of Universiti Putra Malaysia and Ministry of Science, Technology and Innovation (MOSTI), project number 02-01-04-SF0370.

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